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Inhibition of Plasma-Mediated Adherence of Sickle Erythrocytes to Microvascular Endothelium by Conformationally Constrained RGD-Containing Peptides

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Adherence of sickle erythrocytes to vascular endothelium likely initiates or participates in microvascular occlusion, leading to ischemic tissue and organ damage characteristic of sickle-cell pain episodes. In vitro, sickle-cell adherence to endothelium involves adhesive plasma proteins and integrin and nonintegrin receptors on sickle cells and endothelial cells. The involvement of arginine-glycine-aspartic acid (RGD) sequences in adhesive plasma proteins and integrin receptors suggests that RGD-containing peptides may inhibit sickle-cell/endothelial-cell adherence. In the present study, inhibition of plasma-mediated sickle-erythrocyte adherence to endothelium using conformationally constrained RGD-containing peptides was quantified in vitro under continuous flow at a shear stress of 1.0 dyn/cm^2. Two conformationally constrained RGD peptides were investigated: 6Z (which has high affinity for $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_{IIb}\beta_3$ integrin receptors), and TP9201 (which preferentially binds to $\alpha_{IIb}\beta_3$). Peptide 6Z at 50 μM inhibited plasma-mediated sickle-cell adherence to microvascular endothelium 70% when incubated with sickle red cells, and 63% when incubated with endothelium. Under similar conditions, peptide TP9201 inhibited plasma-mediated sickle-cell adherence up to 85% at concentrations from 250 to 500 μM TP9201. The inhibition of plasma-mediated adherence by conformationally constrained RGD peptides, but not by linear or circular constructs, suggests that the tertiary structure of the peptide containing the binding sequence is important. Inhibition of plasma-mediated sickle-cell adhesion with these peptides in vitro suggests that such conformationally constrained RGD peptides could provide therapeutic interventions in the course of the disease by inhibiting receptor-ligand interactions.

Key words: sickle-cell adhesion, sickle erythrocytes, plasma, integrins, RGD peptides

INTRODUCTION

The abnormal adherence of sickle erythrocytes to vascular endothelium may initiate or contribute to the pathophysiology of vascular occlusion [1,2], although the primary problem is polymerization of sickle hemoglobin. Sickle erythrocyte adhesion may initiate occlusion by retarding blood flow, increasing capillary transit time, and hence allowing intracapillary sickling to occur [3,4]. The subsequent entrapment of dense, nondeformable sickle cells may facilitate the propagation of the infarctive crisis [5], resulting in ischemic tissue and organ damage characteristic of sickle-cell anemia [6,7]. Adherence is posited to occur in the postcapillary venules, where shear stress is low [8]. However, under some conditions, adhesion to capillary endothelium may also be significant [9].

Both membrane abnormalities and increased adhesogenic plasma factors contribute to the adherence of sickle erythrocytes in vitro [10]. Recent studies have identified several biochemical mechanisms of sickle-cell/endothelial-cell adherence (reviewed in Wick and Eckman [11]): high molecular weight von Willebrand factor (HMWvWF) multimers bridging GPIb-like and integrin receptors on endothelial cells and similar receptors on sickle cells [12,13]; thrombospondin bridging CD36 on

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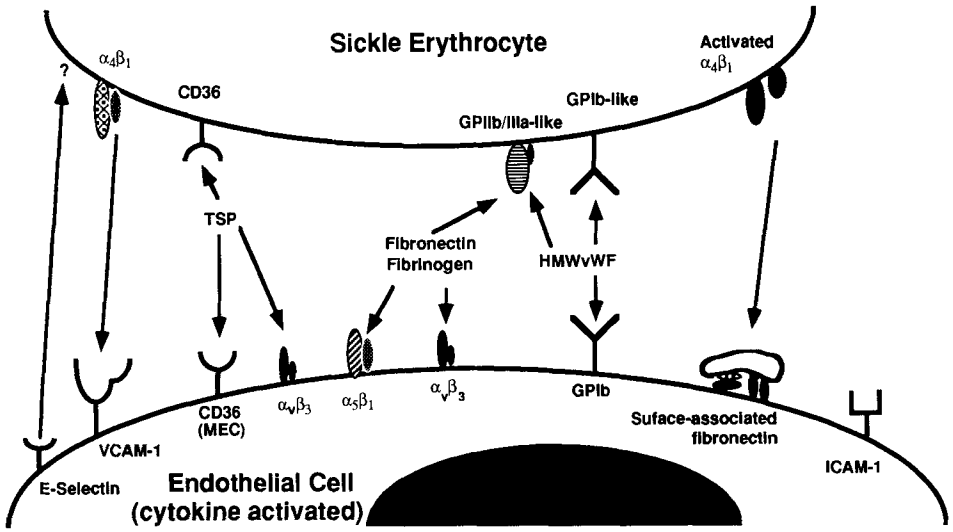

Fig. 1. Sickle-cell/endothelial-cell adherence pathways. Ligands and receptors identified in sickle-cell/endothelial-cell adherence are summarized. Additional details are provided in references as cited in text [11–19].

sickle reticulocytes [14], and CD36 and $\alpha_v\beta_3$ on microvascular endothelium [15] or $\alpha_v\beta_3$ on large-vessel endothelium [14]; $\alpha_4\beta_1$ on sickle reticulocytes [16–18] binding to vascular cell adhesion molecule-1 (VCAM-1) on cytokine-stimulated endothelium [16]; chemokine-activated $\alpha_4\beta_1$ on sickle reticulocytes binding to fibronectin associated with the endothelial cell surface [19]; and sickle cells binding to E-selectin on cytokine-activated endothelium via as-yet unidentified ligands [20]. These adherence pathways are summarized in Figure 1. It is clear that several distinct integrin receptors and their ligands may simultaneously participate in sickle-cell adherence in vivo.

Integrins are a family of αβ heterodimeric transmembrane glycoprotein adhesion receptors, involved in cell-cell and cell-matrix interactions [21–23]. They bind to a number of extracellular matrix proteins including fibrinogen, fibronectin, von Willebrand factor (vWF), vitronectin, collagen, laminin, and thrombospondin [23]. The recognition site for integrins usually includes the Arg-Gly-Asp (RGD) tripeptide sequence. The surrounding amino acids confer specificity for a specific integrin.

The sickle-cell adherence pathways described above have generally been studied in purified systems that isolate individual proteins or adhesion receptors. These have been helpful in characterizing adhesion mechanisms. However, in vivo, multiple and different pathways are likely involved in sickle-cell adherence, and the relative importance of a given pathway may be partially dependent on endothelial-cell phenotype [9].

In purified adherence systems, a linear RGD peptide (RGDS) inhibits sickle red-cell adherence mediated by high molecular weight vWF [13]. Peptide RGDS also blocks adherence of sickle erythrocytes potentiated by thrombospondin [14]. Additionally, adhesion by the two mechanisms involving $\alpha_4\beta_1$ on sickle reticulocytes (see Fig. 1) is inhibited by RGD peptides in our studies (unpublished observations). Hence, peptides reproducing integrin-binding sites may provide the basis for novel therapeutic agents by inhibiting or reversing integrin-mediated sickle-cell adhesion. However, despite the involvement of several integrin receptors in adhesion, plasma-mediated adhesion of sickle erythrocytes to the endothelium could not be blocked by monoclonal antibodies, a linear RGD peptide (RGDS), or a commerically-available cyclic RGD peptide (G(Pen)GRGDSPCA) in a previous study [9,24]. This is likely because monoclonal antibodies and the RGD constructs used were incapable of preventing access to all of the integrin receptors involved in sickle-cell adherence in the plasma milieu.

In the present study, we quantified the ability of two conformationally constrained RGD peptides, 6Z (G(Pen)RARGDNPCA) and TP9201 (Ac-CNPRGD(Y-OMe)RC-NH_2), to inhibit plasma-mediated adhesion of sickle cells to endothelial cells. These are cyclic disulfide peptides, and their structures are published elsewhere [25]. Peptide TP9201 is highly potent and selective for $\alpha_{IIb}\beta_3$ in platelet aggregation and enzyme-linked immunosorbent assay (ELISA) studies. Peptide 6Z shows a high affinity for $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$ in ELISA studies [25,26].

MATERIALS AND METHODS

Endothelial Cell Culture

Human dermal microvascular endothelial cells (HDMEC) isolated from neonatal foreskins were gener-

ously provided by Dr. Robert A. Swerlick, Department of Dermatology, Emory University School of Medicine, Atlanta, GA. HDMEC were cultured in endothelial basal medium (MCDB 131; Gibco Laboratories, Inc., Grand Island, NY) plus 30% (v/v) human AB serum (Gibco), 0.292 mg/ml L-glutamine (Sigma Chemical Co., St. Louis, MO), 0.227 mg/ml cyclic AMP (Sigma), 0.1 mg/ml streptomycin, penicillin, and amphotericin (PSF; Gibco), 0.001 mg/ml hydrocortisone (Sigma), and 0.01 mg/ml epidermal growth factor (EGF; Clonetics Corporation, San Diego, CA), as previously described [9].

Confluent HDMEC were subcultured 1:3 using 0.01% trypsin EDTA (Gibco) and used in assays between passages 2–5. For adherence assays, HDMEC were grown in 0.1% gelatin (Sigma)-coated cell culture chambers (single-well Permanox chamber slides, Nunc, Inc., Naperville, IL), and fed with fresh medium every second day. Monolayers were used within 2 days of reaching confluence.

Reagents for Adherence Assays

Peptides 6Z, TP9201, and 7R were generously made available by Dr. Juerg Tschopp, Telios Pharmaceuticals Inc., San Diego, CA. Lyophilized, sterile peptides were reconstituted in sterile water. Peptide TP9201 is highly potent and selective for $\alpha_{IIb}\beta_3$ in platelet aggregation and ELISA studies. Peptide 6Z shows a high affinity for $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$,and $\alpha_V\beta_3$ in ELISA studies [25,26]. Control peptide 7R has low affinity for $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$, and $\alpha_V\beta_5$.

Red Blood Cell Suspensions

Blood was drawn from patients with homozygous (SS) sickle-cell anemia and from normal donors after obtaining written informed consent at the Georgia Comprehensive Sickle Cell Center at Grady Memorial Hospital, Atlanta, GA. This study was approved by the Insitutional Review Boards of the Georgia Insititute of Technology, Emory University School of Medicine, and Grady Memorial Hospital. All research was performed according to the principles of the Declaration of Helsinki. The blood was centrifuged at 100*g* for 10 min at 25°C. The plasma and buffy coat were removed and the erythrocytes were washed twice in Dulbecco's phosphate-buffered saline (DPBS; Sigma) supplemented with 0.2% (w/v) human albumin (Sigma), 5.0 μg/ml human transferrin (Sigma), and 5.0 μg/ml bovine insulin (Sigma). Platelet-poor plasma was obtained by centrifuging platelet-rich plasma at 400*g* for 15 min. Heparin (Sigma) at a concentration of 15 U/ml was added to prevent coagulation. Platelet-poor plasma was diluted to 30 volume percent with serum-free medium (SFM; microvascular endothelial cell culture medium without serum and supplemented with 0.2% (w/v) human albumin, 5.0 μg/ml human transferrin, and 5.0 μg/ml bovine insulin) for use in adherence assays [9]. Washed erythrocytes were suspended to 1% hematocrit in perfusion medium (SFM) or SFM-containing 30% plasma for adhesion assays.

In order to test the ability of RGD peptides to inhibit plasma-mediated adherence, packed red cells were incubated with peptide for 1 hr at room temperature. The pellet was then resuspended in the 30% plasma suspension to give 2 ml of 1% suuspension (volume used in each assay), and used in the adherence assay. In other experiments, peptide was incubated with HDMEC that had been rinsed twice with Medium 199 (Sigma), for 1 hr at 37°C. Peptide 6Z was used at 50 μM, and peptide TP9201 at 500 μM, unless otherwise noted.

Adhesion Assays

The parallel plate flow chamber used in the present assays has been previously described [12,15,24]. The flow chamber was assembled on the stage of an inverted phase-contrast microscope (Diaphot, Nikon, Japan). The shear stress was held constant at a postcapillary venule shear stress of 1.0 dyne/cm^2 using a syringe pump (Harvard Apparatus, South Natick, MA), and temperature was maintained at 37°C with the aid of a water bath and an air curtain incubator (Nicholson Precision Instruments, Bethesda, MD).

Briefly, endothelial cells were rinsed for 5 min with SFM to remove adhesive proteins secreted during culture. Red cells were then perfused over the endothelial monolayer for 10 min. Nonadherent red cells were removed by a 20-min rinse with SFM. While the endothelial cells continued to be perfused with rinse medium, adherent red cells were counted in 20 randomly chosen fields. The average number of adherent cells per field was normalized to the number of adherent cells per mm^2 and counted as a single observation.

An experiment consisted of a series of adherence assays on endothelial cells from the same culture and red cells from the same donor. In each experiment, sickle-cell adherence to endothelium was measured when sickle cells were suspended in serum-free medium and when sickle cells were suspended in serum-free medium containing 30% autologous plasma. In order to quantify the ability of a peptide to inhibit plasma-mediated adherence, the peptide was incubated with washed sickle cells or with rinsed endothelium prior to measuring adherence in 30% autologous plasma. In this way, the observed inhibition of adherence could be attributed to the presence of the peptide antagonist. Percent inhibition by peptide was defined as:

$$\%\ \text{adhesion inhibition} = \left\{ 1 - \frac{\text{SRBC/mm}^2\ \text{in (plasma + peptide)} - \text{SRBC/mm}^2\ \text{in SFM}}{\text{SRBC/mm}^2\ \text{in plasma} - \text{SRBC/mm}^2\ \text{in SFM}} \right\}$$

TABLE I. IC_{50} Values for Peptides 6Z, TP9201, and 7R*

Peptide	Plt. Aggr.	$\alpha_{IIb}\beta_3$	$\alpha_5\beta_1$	$\alpha_V\beta_3$	$\alpha_V\beta_5$	Molecular weight
6Z	58.0	0.03	0.0021	0.052	0.33	1,145.5
TP9201	0.22	0.021	8.7	0.38	6.1	1,136.5
7R	200.0	14.5	10.0	ND	10.0	1,380.9

*Concentrations for 50% inhibition (IC_{50}, μM) of platelet aggregation or ELISA binding are presented for peptides 6Z, TP9201, and 7R, as described [25,26]. Platelet aggregation studies were done in platelet-rich plasma with 10 mM ADP. Integrin receptor assays for $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$ were ELISA assays with fibrinogen and fibronectin, respectively, as ligands. Integrin $\alpha_V\beta_5$ and $\alpha_V\beta_3$ were ELISA assays, with vitronectin as ligand. These data were provided by Dr. Juerg Tschopp, Telios Pharmaceuticals, Inc., San Diego, CA. ND, not determined.

Each experiment was repeated several times using blood samples from different patients and HDMEC from different cultures. Mean ± SEM calculated using the pooled variance of several experiments is reported in each case. Paired Student's t-tests were used to determine the significance of differences between the means of treatments of grouped data [27].

RESULTS

Table I summarizes the mean concentration of peptide required to inhibit 50% (IC_{50}) of platelet aggregation or ELISA binding as described by Cheng et al. [25]. Additional data were provided by Dr. Juerg Tschopp, Telios Pharmaceuticals. Peptide TP9201 exhibits a strong specificity for $\alpha_{IIb}\beta_3$, whereas peptide 6Z has affinity toward all the integrin receptors tested ($\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$, and $\alpha_V\beta_3$) (Table I). Peptide 7R does not have high affinity for any of the receptors tested and was used as a negative control.

Adhesion of sickle erythrocytes in 30% autologous plasma to microvascular endothelium averaged 51.6 ± 1.6 adherent cells/mm^2 as compared to 1.7 ± 0.3 cells/mm^2 in serum-free medium (mean ± SEM, n = 10, $P = 0.02$). Preincubation of sickle erythrocytes with peptide 6Z at 50 μM inhibited plasma-mediated sickle-cell adherence by an average of 70% (Table II). Peptide 6Z on the endothelium inhibitied plasma-mediated sickle-cell adherence by an average of 63% (Table II).

Plasma-mediated sickle-cell adherence to endothelium was inhibited up to 85% by peptide TP9201, with optimal concentration between 250–500 μM (Fig. 2). Increases in peptide TP9201 up to 2.5 mM did not further inhibit plasma-mediated adhesion (data not shown). For additional experiments, peptide TP9201 was used at 500 μM. In seven experiments, plasma-mediated sickle-cell adherence was inhibited 43–85% when the peptide was incubated with sickle cells, with an average inhibition of 67% (Table II). Similarly, when endothelial cells were incubated with peptide TP9201, plasma-mediated sickle-cell adherence was inhibited 72% and 80% in two experiments (Table II). Percent inhibition by peptide TP9201 did not correlate with plasma adherence level (data not shown).

Peptide 7R, which does not inhibit platelet aggregation or have high affinity for any of the integrins tested (Table I), was used as a negative control at 500 μM. Peptide 7R did not inhibit plasma-mediated sickle-cell adherence to endothelium when incubated with sickle erythrocytes (Table II) under conditions where peptides 6Z and TP9201 were observed to inhibit sickle-cell adhesion.

DISCUSSION

Adhesion of sickle erythrocytes to vascular endothelium is believed by some to initiate sickle-cell vasoocclusive pain episodes [1,2]. Specific adhesion pathways have been characterized using in vitro adherence assays (Fig. 1). Adhesion receptors have been identified on sickle erythrocytes by immunofluorescence [14,16–18], and the role of sickle-cell receptors in adherence has been inferred by blocking studies with antibodies or peptide antagonists [12–16,18,19]. Several adherence pathways involve integrin receptors (Fig. 1). In general, these adherence pathways have been identified under isolated conditions, where only one adherence mechanism could be invoked. However, in vivo, multiple and different adherence pathways are likely to be active in the plasma milieu. In the present study, adherence assays were performed with sickle cells suspended in 30% autologous plasma to more accurately mimic the biochemical and hemodynamic conditions in the microcirculation. Under these conditions, two different peptides were found to inhibit up to 84% of plasma-mediated sickle-cell adherence to human microvascular endothelium under shear-flow conditions. The inhibition of plasma-mediated adhesion by RGD peptides shown here suggests that integrin receptor pathways contribute significantly to the adherence of sickle cells to endothelium mediated by autologous plasma.

Peptide 6Z was found to be maximally effective at 50 μM concentration. In contrast, peptide TP9201 could only inhibit plasma-mediated sickle-cell adherence to microvascular endothelium at 250–500-μM concentration (Fig. 2). The affinity of TP9201 for most integrin receptors (with the exception of $\alpha_{IIb}\beta_3$) is much lower than for peptide 6Z (Table I). Thus, TP9201 is less efficient at competing with plasma-adhesion proteins for integrin receptor-binding sites on sickle cells or endothelial cells, and greater amounts of TP9201 are required to achieve maximal inhibition of plasma-mediated sickle-cell adherence. These observations suggest that development of peptides with high affinity for several integrin receptors may be most effective at inhibiting sickle-cell adherence in the microvascular milieu.

Although the average 67% inhibition of sickle-cell adherence observed with TP9201 at 500 μM did not reach statistical significance ($P = 0.06$), the large percent inhi-

TABLE II. Peptide Inhibition of Plasma-Mediated Sickle-Cell Adherence†

Peptide	Peptide added to:	Sickle-cell suspension medium: SFM	Plasma	Plasma + peptide	Percent inhibition
6Z (n = 7)	SRBC	1.9 ± 0.2	66.5 ± 1.5	21.0 ± 1.0*	70%
6Z (n = 4)	HDMEC	2.3 ± 0.3	33.7 ± 1.7	14.1 ± 0.8*	62%
TP9201 (n = 7)	SRBC	1.8 ± 0.3	58.9 ± 1.6	20.6 ± 1.1**	67%
TP9201 (n = 2)	HDMEC	4.9	30.8	10.2	80%
		1.8	67.3	20.2	72%
7R (n = 3)	SRBC	1.2 ± 0.2	110.2 ± 1.7	98.0 ± 1.9	

†Data are mean ± SEM adherent sickle cells under indicated condition for n different patients, except that for blocking with peptide TP9201 on HDMEC, the normalized adherence is shown for each of two experiments. Sickle erythrocytes were suspended in serum-free medium (SFM, not containing adhesive proteins or agonists), 30% autologous plasma in SFM (plasma), or 30% autologous plasma in SFM after incubation of sickle red-blood cells (SRBC) or microvascular endothelial cells (HDMEC) with peptide, as described in Materials and Methods.

*Inhibition by peptide is statistically significant at $P < 0.05$. For some donors, several conditions were tested. In total, 10 different patients were studied for the data in Table II.

**$P = 0.06$.

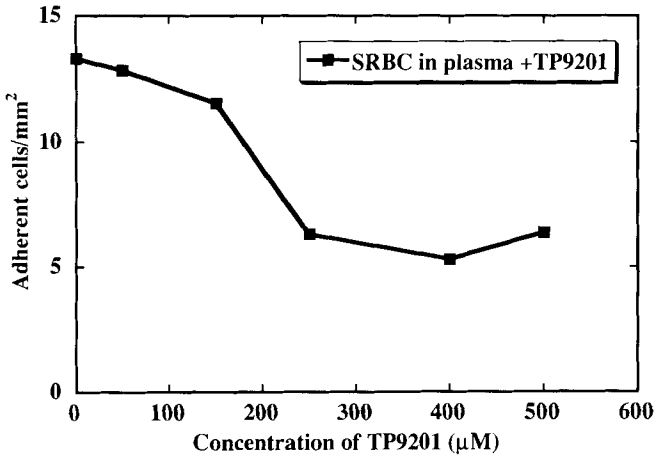

Fig. 2. Inhibition of plasma-mediated sickle-cell adherence with peptide TP9201. Data are from one of three similar experiments using sickle cells from different donors, demonstrating that the optimal inhibitory concentration of peptide TP9201 is between 250–500 μM.

bition in each of the seven experiments (Table II) indicates that this peptide is consistently effective at inhibiting plasma-mediated sickle-cell adherence. In these experiments, plasma-mediated adherence ranged from 15.7 ± 3.3–204 ± 11 red cells/mm² (mean ± SD for 20 fields of view on each endothelial monolayer), and was inhibited 43–84% when peptide TP9201 was incubated with sickle red cells. The lack of statistical significance in peptide-blocking is likely due to the large range of plasma-mediated adherence, which reduces the statistical power of the data set [9]. The fact that inhibition with peptide TP9201 was observed in all experiments, in spite of this variance, demonstrates that peptide TP9201 is consistently effective at blocking sickle-cell adherence mediated by plasma.

Since multiple adherence pathways are likely active in the plasma milieu in vivo, it is not feasible to block all possible adherence pathways by using single monoclonal antibodies. Alternatively, targeting a whole class of receptors, such as integrins, may provide more effective blocking of sickle red-cell adhesion in plasma. Inhibition achieved in this work, by conformationally constrained RGD peptides, and not by other linear or cyclic constructs as in other studies [9], suggests that identification of the conformation of the residues necessary for binding is essential to biologically potent and receptor-selective peptide design. We hypothesize that linear or unconstrained RGD peptides may not bind to the integrin receptors involved in sickle-cell adherence with an affinity high enough to inhibit the binding of the various adhesive proteins in plasma, even though they inhibit adherence to endothelium mediated by purified proteins.

Synthetic peptides corresponding to the recognition sequences of receptors mimic the activity of intact protein ligands. However, peptides alone do not always bind with the same affinity as the native protein. It appears that additional information is supplied by the intact protein molecule. To further complicate matters, in the complex plasma environment, several adhesive proteins may act in concert with each other. Synthetic RGD-containing peptides can be designed to exhibit varying integrin specificities by utilizing conformational constraints [26]. These would force RGD-containing analogues into a conformation akin to that required for productive peptide/receptor interactions [25].

Integrins have the ability to distinguish among the various ligand proteins, even though perhaps all of them have the RGD cell attachment site. One explanation for this could be that the RGD sequence serves as a shared binding site, and a second binding site, unique to each protein ligand, generates specificity [28,29]. Alterna-

tively, the surrounding sequences force the RGD determinant into a unique conformation [28,29]. Cheng et al. [25] suggest the following hypotheses for the specificity of interactions between integrin receptors and RGD peptides. The RGD tripeptide sequence contains all the information required for interaction with a specific RGD-directed adhesion receptor. The surrounding sequences may then force the RGD peptides into an appropriate conformation, hence providing it specificity for a particular receptor. Alternatively, the side chains or backbone elements of the residues flanking the RGD sequence may play a more active biological role in influencing receptor affinity and selectivity. Adhesion receptors appear to recognize differences in the conformation and environment of the RGD tripeptide [26]. This may explain the effectiveness of conformationally constrained peptides 6Z and TP9201 in the inhibition of sickle-cell adhesion vs. the linear and cyclic RGD peptides used by Brittain et al. [9].

Inhibition of sickle-erythrocyte adherence to vascular endothelium may be a viable strategy for therapeutic intervention in the course of episodic sickle-cell microvascular occlusion and pain, using therapies based on interfering with receptor-ligand interactions and cell-adhesion molecule expression. More effective and practical ways of intervening than through the use of monoclonal antibodies, such as with peptides, may well be the outlook for the future [30]. The inhibition of plasma-mediated sickle-erythrocyte adherence to microvascular endothelium by the conformationally constrained peptides 6Z and TP9201, with structures containing the RGD sequence, is encouraging in that it could form the basis of antiadhesion therapies, especially since inhibition of sickle adherence in plasma has not been previously reported.

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